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Fabrication of Fucoxanthin-Loaded Microsphere (F-LM) By Two Steps Double-Emulsion Solvent Evaporation Method and Characterization of Fucoxanthin before and after Microencapsulation

Dedi Noviendri[1], Irwandi Jaswir[1, 2, 3,*], Muhammad Taher[4], Farahidah Mohamed[4], Hamzah Mohd Salleh[1], Ibrahim Ali Noorbatcha[1], Fitri Octavianti[5], Widya Lestari[6], Ridar Hendri[7], Hasna Ahmad[1], Kazuo Miyashita[8] and Alias Abdullah[9]

[1] *Bioprocess and Molecular Engineering Research Unit (BPMERU), Department of Biotechnology Engineering, Faculty of Engineering, International Islamic University Malaysia (IIUM) Gombak, Kuala Lumpur, MALAYSIA*
[2] *International Institute for Halal Research and Training (INHART) International Islamic, University Malaysia (IIUM) Gombak, Kuala Lumpur, MALAYSIA*
[3] *Marine Natural Products Research Centre, Surya University, Tangerang, INDONESIA*
[4] *Department of Pharmaceutical Technology, Faculty of Pharmacy, International Islamic University Malaysia Kuantan, MALAYSIA*
[5] *Faculty of Dentistry, Universiti Sains Islam Malaysia, Kuala Lumpur, MALAYSIA*
[6] *Faculty of Denstistry, International Islamic University Malaysia Kuantan, MALAYSIA*
[7] *Faculty of Fisheries, Riau University, Pekanbaru, INDONESIA*
[8] *Faculty of Fisheries Sciences, Hokkaido University, Hakodate, JAPAN*
[9] *Faculty of Architecture and Environmental Design, International Islamic University Malaysia (IIUM) Gombak, Kuala Lumpur, MALAYSIA*

Abstract: Microencapsulation is a promising approach in drug delivery to protect the drug from degradation and allow controlled release of the drug in the body. Fucoxanthin-loaded microsphere (F-LM) was fabricated by two step w/o/w double emulsion solvent evaporation method with poly (L-lactic-co-glycolic acid) (PLGA) as carrier. The effect of four types of surfactants (PVA, Tween-20, Span-20 and SDS), homogenization speed, and concentration of PLGA polymer and surfactant (PVA), respectively, on particle size and morphology of F-LM were investigated. Among the surfactants tested, PVA showed the best results with smallest particle size (9.18 μm) and a smooth spherical surface. Increasing the homogenization speed resulted in a smaller mean F-LM particle size [*d*(0.50)] from 17.12 to 9.18 μm. Best particle size results and good morphology were attained at homogenization speed of 20 500 rpm. Meanwhile, increased PLGA concentration from 1.5 to 11.0 (% w/v) resulted in increased F-LM particle size. The mean particle size [*d*(0.5)] of F-LM increased from 3.93 to 11.88 μm. At 6.0 (% w/v) PLGA, F-LM showed the best structure and external morphology. Finally, increasing PVA concentration from 0.5 to 3.5 (% w/v) resulted in decreased particle size from 9.18 to 4.86 μm. Fucoxanthin characterization before and after microencapsulation was carried out to assess the success of the microencapsulation procedure. Thermo gravimetry analysis (TGA), glass transition (*Tg*) temperature of F-LM and fucoxanthin measured using DSC, ATR-FTIR and XRD indicated that fucoxanthin was successfully encapsulated into the PLGA matrix, while maintaining the structural and chemical integrity of fucoxanthin.

Key words: fucoxanthin, microsphere, microencapsulation, w/o/w double emulsion, solvent evaporation method

1 Introduction

Microencapsulation (ME) has been defined as the technology of packaging or coating of solid, gaseous and liquid materials with a thin protective layer or wall material[1)] or in small capsules that release their contents at controlled rates over prolonged periods of time[2)]. ME is one of the

*Correspondence to: Irwandi Jaswir, Bioprocess and Molecular Engineering Research Unit (BPMERU), Department of Biotechnology Engineering, Faculty of Engineering, International Islamic University Malaysia (IIUM) Gombak, 53100 Kuala Lumpur, MALAYSIA
E-mail: irwandi@iium.edu.my
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most interesting modes of drug delivery systems[3]. Furthermore, ME in biodegradable polymers, (e.g. PLGA), was found to be a promising approach to protect potential drugs from rapid degradation[4].

PLGA is most commonly used as a drug carrier due to its biodegradability and mechanical strength[5]. It is most popular among the various synthetic biodegradable polymers[6] for pharmaceutical[7] and biomedical applications[8], because it is biocompatible[10, 11], and has been approved by the American Food and Drug Administration (FDA) for clinical usage[9]. PLGA is also popular because of its ability to control the release of bioactive macromolecules[12], its high versatility[11], favorable degradation characteristics and possibilities for sustained drug delivery systems[9].

Solvent extraction/evaporation method is the most popular technique of preparing PLGA microspheres or microparticles[13, 14], and this technique has been extensively studied in recent years for the preparation of microspheres[15]. The solvent/ extraction concept employs methylene chloride or dichloromethane (DCM) and water as dispersed and continuous phase, respectively, in an emulsion type system[16]. This method does not require phase separation- inducing agents or elevated temperatures[8, 17], and could be easily scaled up to produce sterile microcapsules[8].

Because of their protection and encapsulation efficiency (EE), w/o/w emulsion is potentially suitable for application in various domains such as cosmetics, foods and pharmaceutics[18]. Thus, in the present study, a fucoxanthin-loaded microsphere (F-LM) was fabricated by two step w/o/w double emulsion solvent evaporation method. The effect of four types of surfactants (PVA, Tween-20, Span-20 and SDS), the homogenization speed, and concentration of PLGA polymer and surfactant (PVA), respectively, on particle size and morphology of F-LM were also investigated.

The properties of fucoxanthin before and after microencapsulation were characterized by laser particle size analyzer, field emission scanning electron microscope (FE-SEM), thermo gravimetry analysis (TGA), differential scanning calorimetry (DSC), attenuated total reflectance-Fourier transform infrared (ATR-FTIR), and X-ray diffraction (XRD).

2 Materials and methods

2.1 Materials

All chemicals used in this study were of analytical grade. The co-polymer PLGA (ratio of lactic to glycolic acid is 50:50; 0.4 dL/g) was purchased from PURAC® (The Netherlands). Polyvinyl alcohol (PVA) with MW 115 kDa was purchased from BDH Laboratory Supplies (England), Tween-20, Span-20 and sodium dodecyl sulfate (SDS) were supplied by MERCK (Germany), fucoxanthin (purity > 95%) was supplied by SIGMA ALDRICH (CAS No. 3351-86-8) and dichloromethane (DCM) was purchased from Fisher Scientific (United Kingdom).

2.2 Fabrication of F-LM

Modified double-emulsion solvent evaporation method was adopted from Mohamed and Walle[19] with some modifications. Briefly, 178 μL of dH_2O was mixed into 22 μL of PVA, producing 0.5% w/v of aqueous phase. This aqueous phase was added into both 120 mg of PLGA (50/50) and 400 μg/mL of fucoxanthin previously dissolved in 2 mL DCM (oil phase). This mixture was homogenized at 20500 rpm (IKA® T10 basic Ultra-Turrax) for 3 minutes (primary emulsion, PE). After homogenization, PE was immediately subjected to 22 mL of 0.5 (% w/v) PVA 10 times the volume of PE[20]. Then, the mixture was homogenized again at 20500 rpm for 10 minutes to produce the secondary emulsion (SE). Subsequently, this SE was transferred into a continuously stirred hardening tank[20] containing 100 mL of 0.5% PVA. This stirring was continued for 2 – 3 hours to allow complete evaporation of DCM. The hardened microspheres were collected by centrifugation (2500 rpm), washed with 600 mL distilled water and then lyophilized overnight[20]. Lyophilized microspheres were kept at −20℃ in an air-tight container with silica gel until further evaluation[20, 21]. Figure 1 shows a schematic diagram of the two-step w/o/w double-emulsion solvent evaporation method of microsphere synthesis[22]. In this study, the fabrication of F-LM was analyzed for the effect of four types of surfactants (PVA, Tween 20, Span 20 and SDS), the homogenization speed, and concentration of PLGA polymer and surfactant (PVA), respectively, on particle size and morphology of F-LM.

2.3 Characterization of F-LM

2.3.1 Particle size analysis

A LPSA (BT-9300H, Better size Instrument Ltd., China), which is a laser diffractometer, was used to determine the size distribution of the microspheres prior to lyophilization. The microspheres were dispersed in water until around 25% obscurity was reached. The size distribution was expressed as volume median diameter (VMD)[19]. Data presented indicate the $d(0.5)$, which is equivalent to volume diameter at 50% cumulative volume[23].

2.3.2 External morphology analysis

FE-SEM (JEOL, JSM 6700F Model) was used to capture images for evaluation of shape, size and external morphology of the microspheres. Briefly, a small amount of lyophilized F-LM was mounted on aluminium stubs pre-pasted with double-sided copper tapes. The samples were sputter-coated with a thin layer of gold and placed inside the specimen chamber at an accelerating voltage of 3 kV at 20℃ and 10^{-5} Torr[20]. The obtained SEM micrographs were examined for characterization of the morphology of the

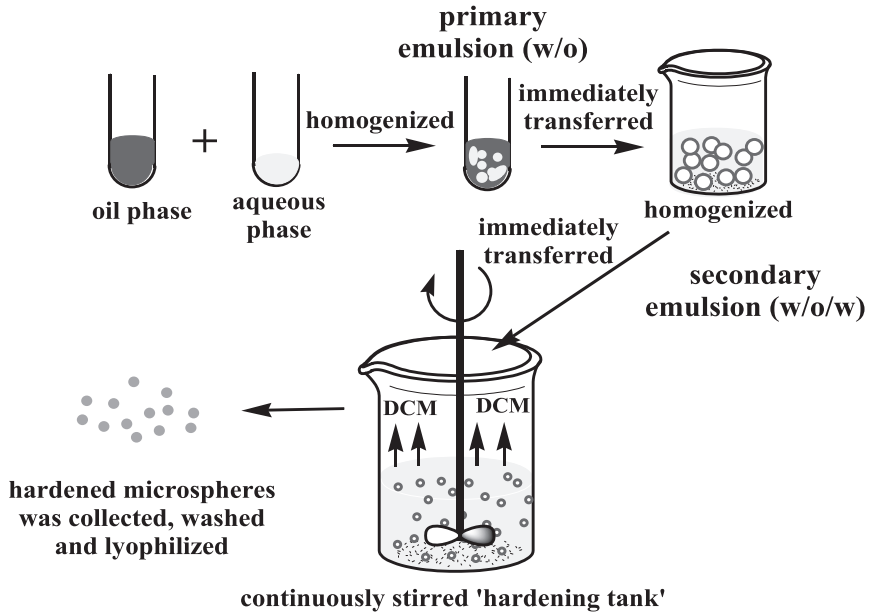

Fig. 1 Schematic diagram of two-step double-emulsion solvent evaporation method of F-LM synthesis.

various microspheres[24].

2.3.3 Thermal behavior analysis by TGA

TGA was performed to study the thermal behavior of fucoxanthin. PLGA (50/50) and F-LM TGA data was obtained using a thermo gravimetric analyzer (PYRIS Diamond TGA, Perkin Elmer). 5 – 10 mg of the samples (fucoxanthin, PLGA and F-LM) were weighed into a sample pan. Measurement was carried out under a nitrogen purge from 39℃ to 650℃, at a heating rate of 10℃ min^{-1} [25].

2.3.4 Glass transition temperature (*Tg*) analysis

In this study, the method of Mohamed and Walle[19] was adopted to measure the *T*g of fucoxanthin (as core), PLGA (50/50) (as coating) and F-LM, respectively. DSC (DSC, Mettler Toledo) with a sensor accuracy of 0.1℃ was used to measure the *T*g of the materials under nitrogen atmosphere. Approximately 2 mg of sample was spread onto a 40 μL aluminium crucible, making sure the edge of the crucible was spill-free of any materials before it was hermetically sealed with a pinhole in the lid[20].

The reference against which the materials were measured consisted of an empty pin-holed aluminium crucible of the same geometry and mass as the sample's crucible[20]. Both the reference crucible and the sample crucible were first allowed to equilibrate at 0℃ for 5 min to ensure isothermal starting conditions. The crucibles were then heated at a rate of 10℃/min from 0 to 85℃, quench cooled to −20℃ and heated again to 85℃ at 10℃/min heating rate[20]. The quench-cooled step was performed to erase any thermal history such as physical ageing that may have occurred during manufacturing. The re-heating was performed immediately at high temperatures (85℃) so that the relaxation time (τ) was minimized to ensure complete molecular relaxation of the sample to equilibrium (Mettler Toledo literature). The bisector method of *T*g determination was used on the scanning curve and calculated using Mettler Toledo STAR® software version 9.10[20].

2.3.5 Functional group analysis

ATR-FTIR spectrometer (Spectrum 100, Perkin Elmer) was used to analyze the functional groups of the samples (fucoxanthin, PLGA and F-LM) by measuring attenuated total reflectance (ATR). ATR-FTIR provides information about the surface composition of the samples depending upon manufacturing procedures and conditions[26].

2.3.6 Crystallinity analysis

The crystallinity of fucoxanthin (as core), PLGA (50/50) (as coating) and F-LM were evaluated using XRD (Shimadzu, XRD-6000 model, Japan) with X-ray tube: Cu (1.54060 A); voltage: 40.0 kV; current: 30.0 mA; step size: 0.0200 deg; and scan range: 30 – 70 deg, 2θ angle.

2.4 Statistical analysis

Statistical analyses were performed using SPSS version 20. All experiments were performed in triplicate and the mean values were reported. Comparison between means was performed with Tukey's test. Differences between means were evaluated as significant at $p \leq 0.05$.

3 Results and discussions

3.1 Effect of surfactants on the F-LM particle size

Four selected surfactants PVA, Tween-20, Span-20 and SDS were incorporated individually into the primary emulsion (PE) and secondary emulsion (SE) during micro-

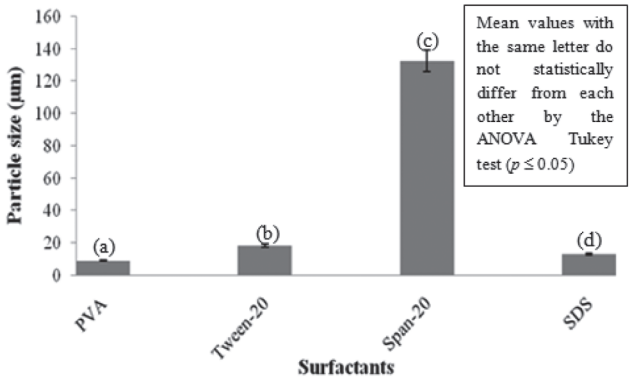

Fig. 2 Effect of surfactants on F-LM particle size.

sphere fabrication. The effect of surfactants on microsphere and size distribution of the F-LM is shown in Figs. 2 and 3. In this study, fabrication of F-LM using PVA as surfactant produced the smallest particle size (9.18 μm), whereas Span-20 produced the largest particle size (132.53 μm). The mean particle size [$d(0.5)$] of F-LM using PVA, Tween-20, Span-20 and SDS as surfactants were 9.18, 18.41, 132.53 and 13.13 μm, respectively. All of the surfactants were found to have significant effects on the overall F-LM particle size as observed in Figs. 2 and 3.

Following the post-hoc Tukey's test ($p \leq 0.05$), significant differences were found between each surfactant. However, F-LM particle size (9.18 μm) with PVA as surfactant was more desirable as a potential candidate for lung cancer treatment. Particle size is crucial in determining potential candidates for lung cancer drug delivery system[27]. Microspheres with particle size >10 μm are not able to penetrate the tracheobronchial tree[15]. The particle size (PS) range is very specific because if they are too large, they do not leave the inhaler. Conversely, if the particles are too small, they will be inhaled[28]. Thus, F-LM fabricated using Tween-20, Span-20 and SDS as surfactants were not desirable due to their PS >10 μm.

The results from observation of external morphologies of F-LM are shown in Fig. 4. Surfactants that generated interesting morphology can be potentially applied as a candidate for lung cancer drug, and thus could be subsequently tested for aerosolisation properties. The external morphology of microspheres produced using PVA as surfactant presented spherical and distinct smooth surfaces (Fig. 4A). However, the external morphology of SDS-derived microspheres was both spherical and nonspherical with discrete smooth surfaces (Fig. 4D). Conversely, the external morphology of microspheres fabricated using Tween-20 and Span-20 as surfactants produced irregular particle shapes that aggregated (Figs. 4B and 4C).

From this study, PVA was found to be the best surfactant, since it produced microspheres with a distinct smooth spherical surface of particle size of around 9 μm. This size is desirable for pulmonary drug delivery (<10 μm). PVA is widely used in the preparation of micro/ nanoparticles[29]. Moreover, PVA is a well-known protective colloid used in

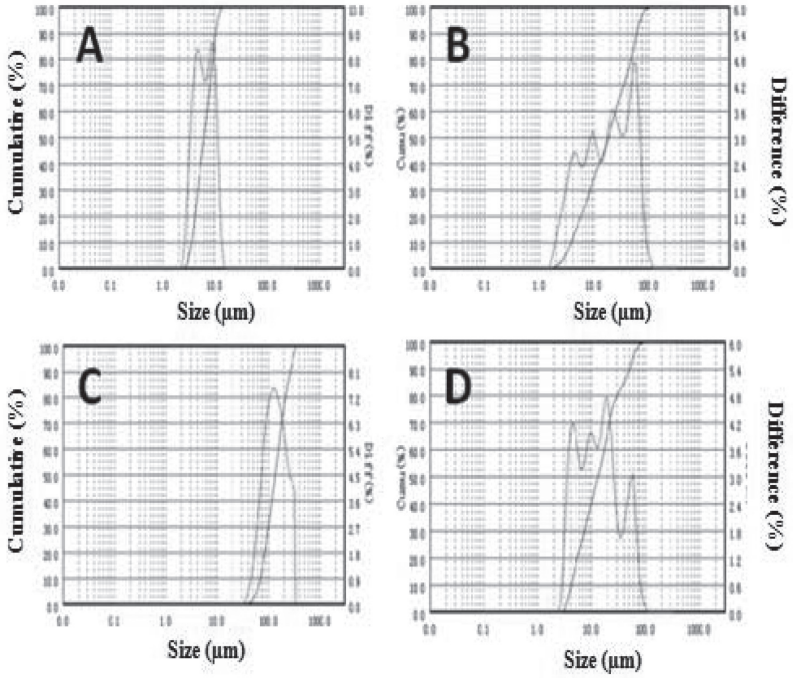

Fig. 3 The effect of surfactants on size distribution of F-LM particle size by using several surfactants incorporated in both primary and secondary emulsion in microsphere fabrication, such as PVA (A), Tween-20 (B), Span-20 (C) and SDS (D). LSPA (BT-9000H, Bettersize Instrument Ltd., China) was used to analyze the size distribution of microspheres.

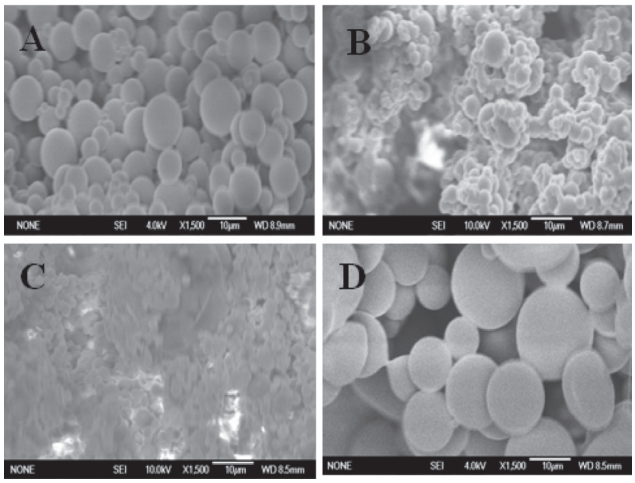

Fig. 4 Scanning electron micrograph (SEM) of microspheres by using surfactants as both primary and secondary emulsion such as PVA (A), Tween-20 (B), Span-20 (C) and SDS (D). The external morphologies of F-LM by using FE-SEM (JEOL, JSM 6700F Model, Japan) with magnification 1500X.

suspension polymerization[30]. The presence of PVA in the external water phase results in smaller emulsion droplets because PVA stabilizes the emulsion droplets against coalescence[10]. The stabilization of the microsphere emulsion with PVA in microspheres occurs with the surface hydroxyl groups[31]. Thus, PVA was used as the surfactant for further studies.

3.2 Effect of homogenization speed on the F-LM particle size

The effects of homogenization speed on microsphere particle size in the fabrication of F-LM are shown in Figs. 5 and 6. In this study, increased homogenization was found to have negative correlation on overall F-LM particle size, as observed in Fig. 5. Thus, increase of homogenization speeds from 8,000 to 20,500 rpm resulted in decreased F-LM particle size. The mean particle size [$d(0.50)$] of F-LM decreased from 17.12 to 9.18 μm.

The mean PS of F-LM with homogenization speeds of 8,000; 11,500; 14,500 and 20,500 rpm were 17.12, 15.12, 12.57 and 9.18 μm, respectively. All of the homogenization speeds were found to have significant effects on the overall F-LM particle size, as can be observed in Fig. 5. Following the post-hoc Tukey's test ($p \leq 0.05$), significant differences were found between homogenization speeds. Zhu *et al.*[32] reported that the particle size of microspheres is mainly dependent on the stirring rate in the emulsion-evaporation method.

Homogenization speed had the largest effect on microsphere size. Increase in homogenization speed produced smaller microspheres[33]. At a higher stirring rate, larger

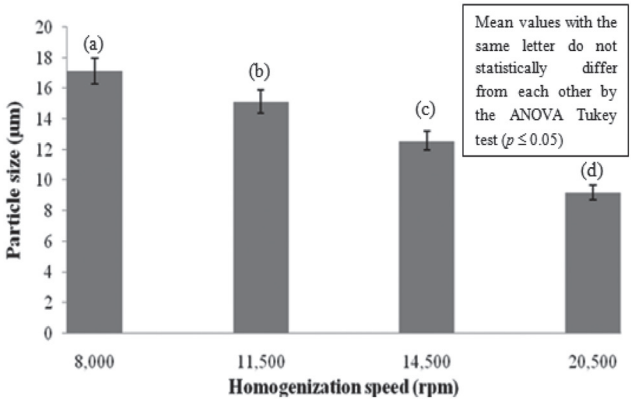

Fig. 5 Effect of homogenization speed on F-LM particle size.

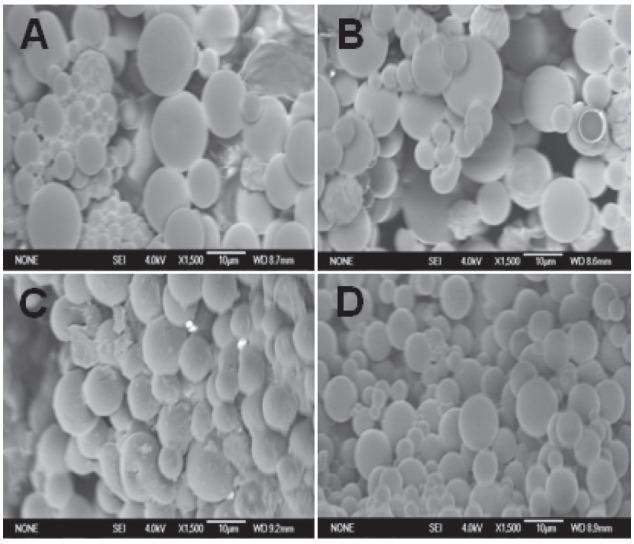

Fig. 6 SEM of F-LM with various of homogenization speed; 8,000 rpm (A); 11,500 rpm (B); 14,500 rpm (C); and 20,500 rpm (D). The external morphologies of F-LM by using FE-SEM (JEOL, JSM 6700F Model, Japan) with magnification 1500X.

shear energy is supplied to separate the oil phase into smaller globules[34]. Hence large surface area can be equilibrated, which corresponds to smaller droplets and particle size[35].

Homogenization speed also affected the external morphology of F-LM (Fig. 6). Figure 6A shows the external morphology of microspheres produced at homogenization speed of 8000 rpm. Several morphologies such as spherical shape, irregular size, discrete particles and aggregates were observed. Aggregates were formed perhaps due to fast diffusion rate of solvent, causing the solvent to diffuse into the aqueous phase before stable microspheres were formed[25]. Particle size was largest at homogenization speed of 8,000 rpm ($d(0.5) = 17.12$). The external mor-

phology of F-LM with homogenization speed of 11,500 rpm (Fig. 6B) was similar to that with speed of 8,000 rpm (Fig. 6A). However, the particle size of F-LM with homogenization speed of 11,500 rpm was lower than that of 8,000 rpm ($d(0.5) = 15.12$ μm). The external morphology of F-LM with homogenization speed of 14,500 rpm was better than both homogenization speeds of 8,000 and 11,500 rpm. The F-LM morphology at this speed was spherical, regular size and discrete with only small aggregates observed. Conversely, the external morphology of F-LM at homogenization speed of 20500 rpm showed spheres with discrete smooth surface (Fig. 6D), and no pores. The particle size of F-LM generated at this speed was the smallest ($d(0.5) = 9.18$ μm).

Ehtezazi *et al.*[36] reported that formulations prepared at higher speed contain smaller and more narrowly distributed pores than those prepared at lower speed. Mehrotra and Pandit[37] reported that high stirring rate enhanced mass transfer and rate of diffusion between the phases, which induced homogenous supersaturation and rapid nucleation to produce smaller sized microspheres. Thus, increasing homogenization speed decreased the size of the microsphere. This is because larger droplets of the dispersed phase are broken down into smaller droplets at high homogenization speeds[38].

3.3 Effect of PLGA concentration on the F-LM particle size

The effects of PLGA concentration on the fabrication of F-LM are shown in Figs. 7 and 8. PLGA concentrations were found to have positive correlation on overall F-LM particle size as observed in Fig. 7. Thus, increased PLGA concentration from 1.5 to 11.0 (% w/v) resulted in increased F-LM particle size. The mean particle size [$d(0.5)$] of F-LM increased from 3.93 to 11.88 μm. The mean particle size of F-LM with PLGA concentrations of 1.5, 3.0, 6.0, 9.0 and 11.0 (% w/v) were 3.93, 6.85, 9.18, 10.95 and 11.88 μm, respectively.

Following the post-hoc Tukey's test ($p \leq 0.05$), significant differences were found between PLGA concentrations. From this study, the effect of PLGA concentration on F-LM particle size was opposite to the effect of homogenization speeds. Fu *et al.*[33] reported that homogenization speed and polymer (PLGA) concentration had the largest effects on microsphere size. Increase in homogenization speeds caused a decrease in the microsphere size, whereas decrease in polymer concentration caused a decrease in microsphere size.

The SEM micrographs of F-LM produced with various PLGA concentrations are shown in Fig. 8. The F-LM produced at all PLGA concentrations showed very smooth and discrete poreless spheres. However, their particle sizes are different. Figure 8A showed that at 6.0 (% w/v) PLGA the structure and external morphology of F-LM was of regular

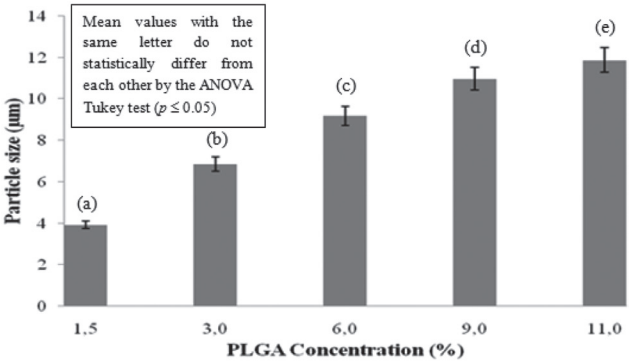

Fig. 7 Effect of PLGA concentration on F-LM particle size.

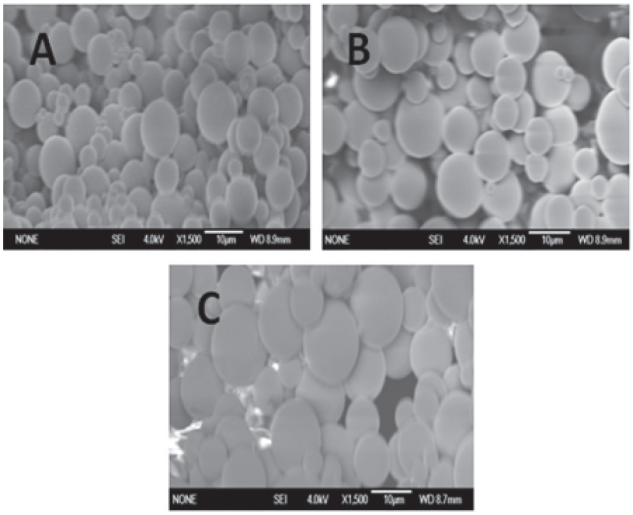

Fig. 8 SEM of F-LM with various PLGA concentrations; 6.0 (% w/v) (A), 9.0 (% w/v) (B) and 11.0 (% w/v) (C). The external morphologies of F-LM by using FE-SEM (JEOL, JSM 6700F Model, Japan) with magnification 1500X.

shape (only spherical), whereas at 9.0 and 11.0 (% w/v), the structures and external morphologies of F-LM were complex and had irregular shapes (spherical and nonspherical) (Figs. 8B and 8C). The complex structures and external morphologies obtained when using higher PLGA polymer concentration were apparent in SEM micrographs of the F-LM in Figs. 8B and 8C. The results can be explained as the coalescence of several microspheres into a single structure, eventually including smaller spheres inside the larger structures[24].

3.4 Effect of PVA concentration on the F-LM particle size

In this study, PVA concentration had profound effects over the particle size surface and external morphology of F-LM (Figs. 9 and 10). PVA concentrations were found to have negative correlation on overall F-LM particle size as observed in Fig. 9. Thus, increasing PVA concentration

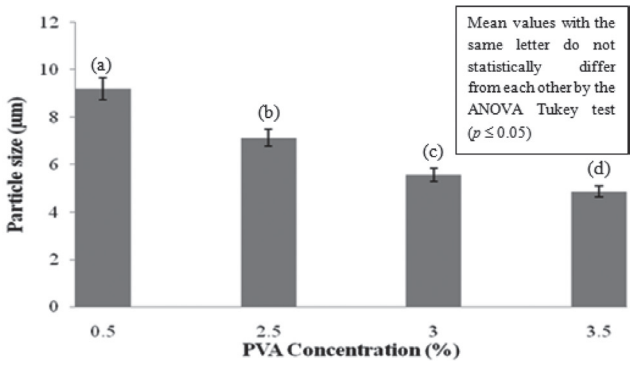

Fig. 9 Effect of PVA concentration on F-LM particle size.

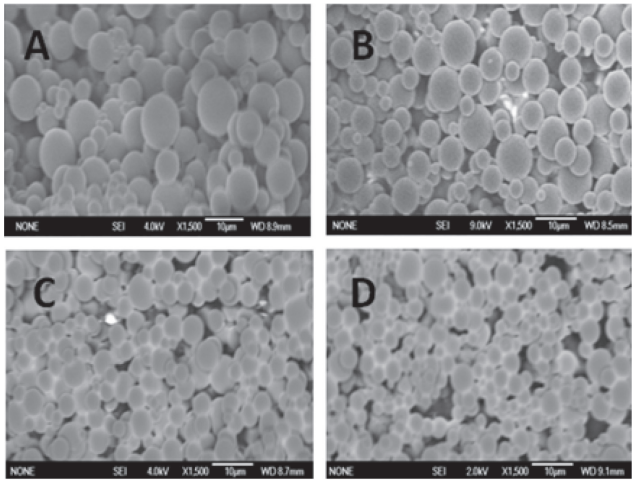

Fig. 10 SEM of F-LM with various PVA concentrations; 0.5 (% w/v) (A), 2.5 (% w/v) (B), 3.0 (% w/v) (C) and 3.5 (% w/v) (D). The external morphologies of F-LM by using FE-SEM (JEOL, JSM 6700F Model, Japan) with magnification 1500X.

from 0.5 to 3.5 (% w/v) resulted in decreased particle size from 9.18 to 4.86 μm. The mean particle size of F-LM with PVA concentration of 0.5, 2.5, 3.0 and 3.5 (% w/v) were 9.18, 7.12, 5.56 and 4.86 μm, respectively. Following the post-hoc Tukey's test ($p \leq 0.05$), significant differences were found between PVA concentrations. Brunner *et al.*[24] reported that increasing the concentration of PVA provides conditions to obtain smaller emulsion droplets resulting in the formation of small microspheres. Increasing the concentration of PVA in the external water phase resulted in smaller emulsion droplets because PVA stabilized emulsion droplets against coalescence[10].

Increasing stabilizer concentration in the external phase decreased the superficial tension between the aqueous and organic phase, which resulted in a reduced diameter size in the multiple emulsion globules[39]. This frequently leads to a decrease in microsphere size[40]. Keegan[31] pointed out that the stabilization of the microsphere emulsion with PVA is due to surface hydroxyl groups.

The SEM micrographs of F-LM with various PVA concentrations are shown in Fig. 10. The F-LM produced at all PVA concentrations showed smooth and poreless surface morphology. However, the particle sizes generated were different.

3.5 Characterization of F-LM

3.5.1 Thermal analysis of materials

The thermal behavior of fucoxanthin, PLGA and F-LM were investigated by thermo gravimetry analysis (TGA) under different heat conditions. TGA measurements provide data regarding the thermal stability of F-LM and weight loss during heating[41]. TGA also provides information on the content of volatile components such as water or solvents, ash or filler content and decomposition behavior of the materials[42].

The TGA curves of fucoxanthin, PLGA and F-LM are shown in Fig. 11. Thermal behavior was investigated up to ~650℃. From 40 to 161℃, fucoxanthin exhibited a 3.4% weight loss. This shows that the decomposition process under heating proceeds in several steps: fucoxanthin evaporation occurs at around 161℃, while PLGA and F-LM degradation start at around 250℃ (weight lost 3.1%), progress with maxima at around 369℃ (weight lost 54.1%) and end at 503.7℃ (weight lost 95.7%). The TGA patterns of PLGA and F-LM were the same, and their curves almost overlapped. The F-LM curve corresponded to the PLGA curve. The results suggest that fucoxanthin was encapsulated inside in the PLGA matrix. In conclusion, no strong chemical interactions that alter the chemical structure and drug structural integrity between fucoxanthin and the PLGA matrix were observed.

3.5.2 The *Tg* analysis of materials

Prasanth *et al.*[43] reported that the thermal analysis of microcapsules/ microspheres and their components can be performed using TGA and DSC. The DSC and TGA curves provide some information about the various observations during heat treatment. The TGA curve reveals the changes in weight, but the DSC, which is associated with calorific phenomenon, occurs with no detectable weight change[44]. DSC has been one of the most widely used calorimetric techniques to characterize and analyze the physical state of drugs encapsulated in the polymeric matrix[45].

In this study, the DSC thermogram of fucoxanthin, PLGA and F-LM are shown in Fig. 12. DSC measures the difference between the heat flow to the sample and the reference pan when subjected to the same temperature program. The heat flow corresponds to transmitted power and is measured in milli watts (mW)[42]. *Tg* is reported as the *T* onset of the corresponding glass transition[8]. *Tg* is exhibited by amorphous polymers or amorphous regions of partially crystalline polymers when a viscous or rubbery phase is transformed into a brittle[46], hard, glass-like phase[22]. *Tg* is one of the crucial properties of any polymer

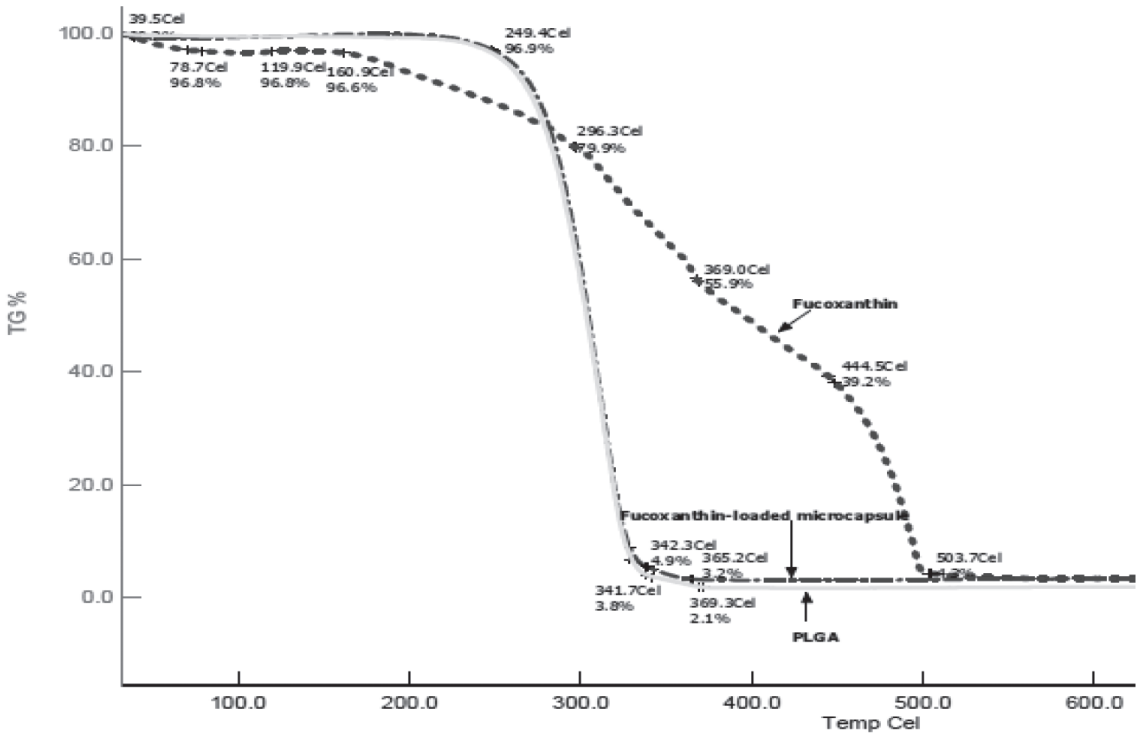

Fig. 11 TGA curve of fucoxanthin, PLGA and FLM. TGA was obtained using a TG analyzer (PYRIS Diamond TGA, Perkin Elmer).

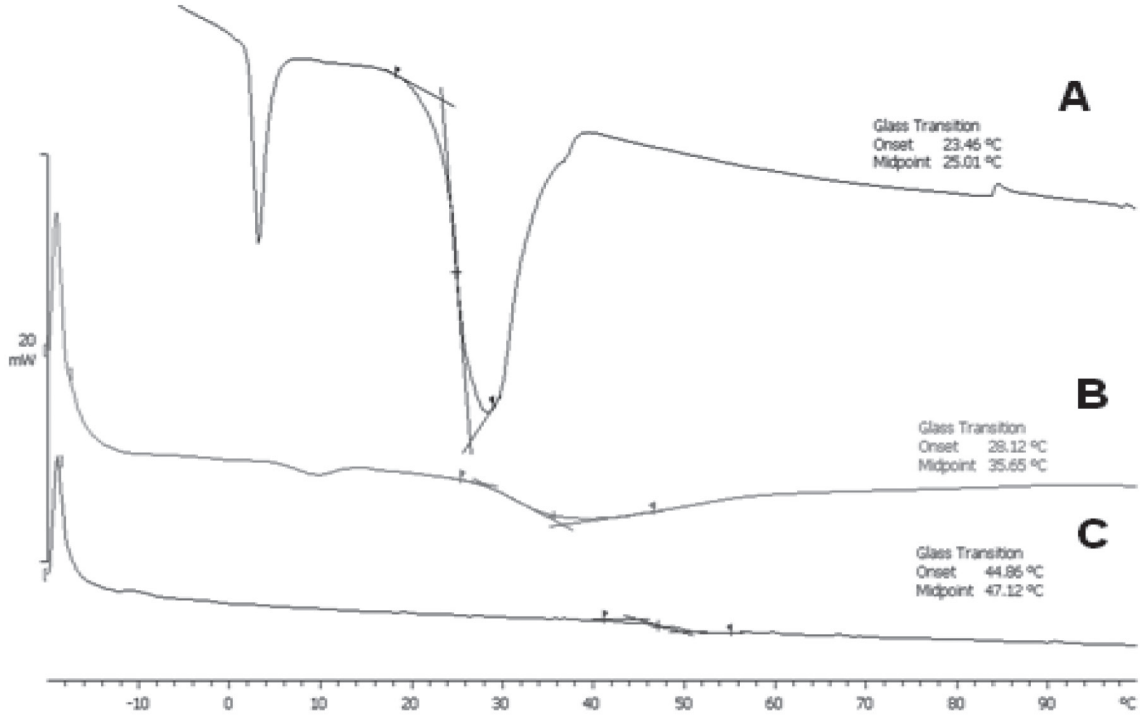

Fig. 12 DSC thermograms of fucoxanthin (A), F-LM (B), and PLGA (C). The *T*g analysis of materials by using DSC (Mettler Toledo STAR® software version 9.10) with sensor accuracy of 0.1℃ under nitrogen atmosphere as the purge gas[20].

or polymer-based substance. This characteristic should be highly considered during development of novel polymer-based products[47].

The *T* onset of PLGA is 44.86℃ (Fig. 12C), indicating that the glass transition temperature of PLGA (50:50) was ~45℃. The *T*g for blank microspheres (44.86℃) corresponded to the *T*g value of raw PLGA, as provided by the manufacturer (ca. 45℃). Wang[48] reported that the *T*g of PLGA (50:50) is between 45 to 50℃.

Moreover, Passerini and Craig[49] reported that the *T*g of PLGA copolymers are commonly above the physiological temperature of 37℃ and hence are glassy at room temperature, thus exhibiting fairly rigid chain structure suitable for controlled drug delivery[50]. A polymer that has a *T*g nearly that of body temperature, when administered, will become more ductile than at room temperature, affecting the mechanical strength to be fabricated into delivery device[15], drug release and degradation[22].

Moreover, F-LM (Fig. 12B) has two endothermic peaks at around 10℃ and 36℃, respectively, whereas the two sharp endothermic peaks of fucoxanthin (Fig. 12A) at 4℃ and 29℃ are due to the fusion and evaporation of the adsorbed water, and the two blunt exothermic peaks at 85℃ and 100℃ are ascribed to the thermal decomposition in consecutive stages during heating. From this study, the *T*g of F-LM and fucoxanthin are 28.12℃ (*T* onset) and 23.46℃ (*T* onset), respectively.

DSC is sensitive to the heat changes associated with thermally-driven phase transition[51]. It is well known that DSC is very helpful to the study of the thermal properties of particles, showing both qualitative and quantitative information on the physicochemical state of the drugs in the particles[52]. DSC also has many applications during pre-formulation screening of new drug candidates, but definitive assignment of peaks to specific events in the sample is difficult without supplementary data from other techniques. Thus, the absence of any chemical interaction between drug and polymer could be confirmed by DSC, FTIR, and TGA techniques. XRD pattern of formulation confirmed the intense superimposition of polymer and drug[25].

3.5.3 Functional group analysis of materials

ATR-FTIR is used to determine the degradation of the polymeric matrix of the carrier system. Additionally, FTIR can be used to characterize the chemical structures of the materials and the interactions between the polymer and the drugs[48]. The surface of the microcapsule/ microsphere is investigated by measuring ATR. The IR beam passing through the ATR cell is reflected many times through the sample to provide the IR spectra of the surface material[26]. ATR-FTIR provides information about the surface composition of the microsphere depending upon manufacturing conditions and procedures[53].

In the present study, fucoxanthin, PLGA matrix and F-LM were assessed by ATR-FTIR, and the obtained spectra are presented in Fig. 13. The PLGA polymer showed peaks such as C-O-C stretching at 1082 cm^{-1} (1050-1250 cm^{-1}), C-H stretching in methyl groups at 1450 cm^{-1}, carbonyl –C=O stretching vibration at 1746 cm^{-1} (1700-1800 cm^{-1}), -CH, -CH_2, -CH_3 stretching vibration at 2955 cm^{-1} (2850-3000 cm^{-1}), and –OH stretching vibration at around 3500 cm^{-1} (3200-3500 cm^{-1}). The pure fucoxanthin sample showed main peaks contributed by the functional groups of molecules such as –CO stretching at 1029 cm^{-1} (1000-1260 cm^{-1}), C-C(O)-C stretching (all others) at 1181 cm^{-1} (1150-1210 cm^{-1}), C-C(O)-C stretching (esters) at 1243 cm^{-1} (1230-1260 cm^{-1}), -CH_3 bend (alkenes) at 1366 cm^{-1} (~1375 cm^{-1}), -C=O stretching (carboxylic acid) at 1722 cm^{-1} (1700-1730 cm^{-1}), -CH stretching at 2931 cm^{-1} (2800-2950 cm^{-1}) and –OH stretching vibration at 3407 cm^{-1} (3200-3500 cm^{-1}).

The spectral analysis indicated that the functional groups of PLGA on the surface of microsphere are almost the same chemical characteristics. On the other hand, there was no significant difference between spectra of physical F-LM and PLGA. No obvious spectrum shift is observed after the formulation of F-LM, which confirms that fucoxanthin had no strong chemical interaction with PLGA and was physically dispersed in the polymeric matrix. The fucoxanthin (as core) maintained good stability during the preparation process. From these characteristic peaks, it can be concluded that fucoxanthin was encapsulated by PLGA as expected. Thus, in this study, the core-polymer interaction and degradation of core during microencapsulation were determined by FTIR[54].

3.5.4 Crystallinity analysis of materials

Crystallinity is defined as the weight fraction of the crystalline portion of a polymer. XRD is most frequently used to measure crystallinity in polymers[44]. The change in crystallinity of a drug can be determined by this technique[43].

In this study, XRD was used to determine crystallinity and polymorphic forms of the samples. The XRD spectra of fucoxanthin, PLGA and F-LM are shown in Fig. 14. Fucoxanthin exhibited several characteristic intense peaks at $2\theta = 37.9^\circ$, 38.5°, 44.2°, 44.6°, 64.5° and 65.1°. All these peaks are ascribed to its crystalline nature (a crystalline solid). For PLGA, there were only quite low peaks. PLGA showed its characteristic low intense peaks at $2\theta = 38.5^\circ$, 44.6° and 65.1°. These peaks are ascribed to PLGA (50/50) because it is more amorphous than fucoxanthin. Yamaguchi *et al.*[55] have reported that PLGA in nature is amorphous.

F-LM in this study showed the same XRD pattern (3 peaks) as PLGA. This correlated with the TGA results (both curves nearly overlapping), and its intense peaks at $2\theta = 38.5^\circ$, 44.6° and 65.1°. F-LM presented peaks of diminishing intensity, suggesting the amorphous nature of fucoxanthin present in the PLGA microsphere. The change in crystallinity of fucoxanthin was determined by this technique. The results indicated that fucoxanthin was successfully en-

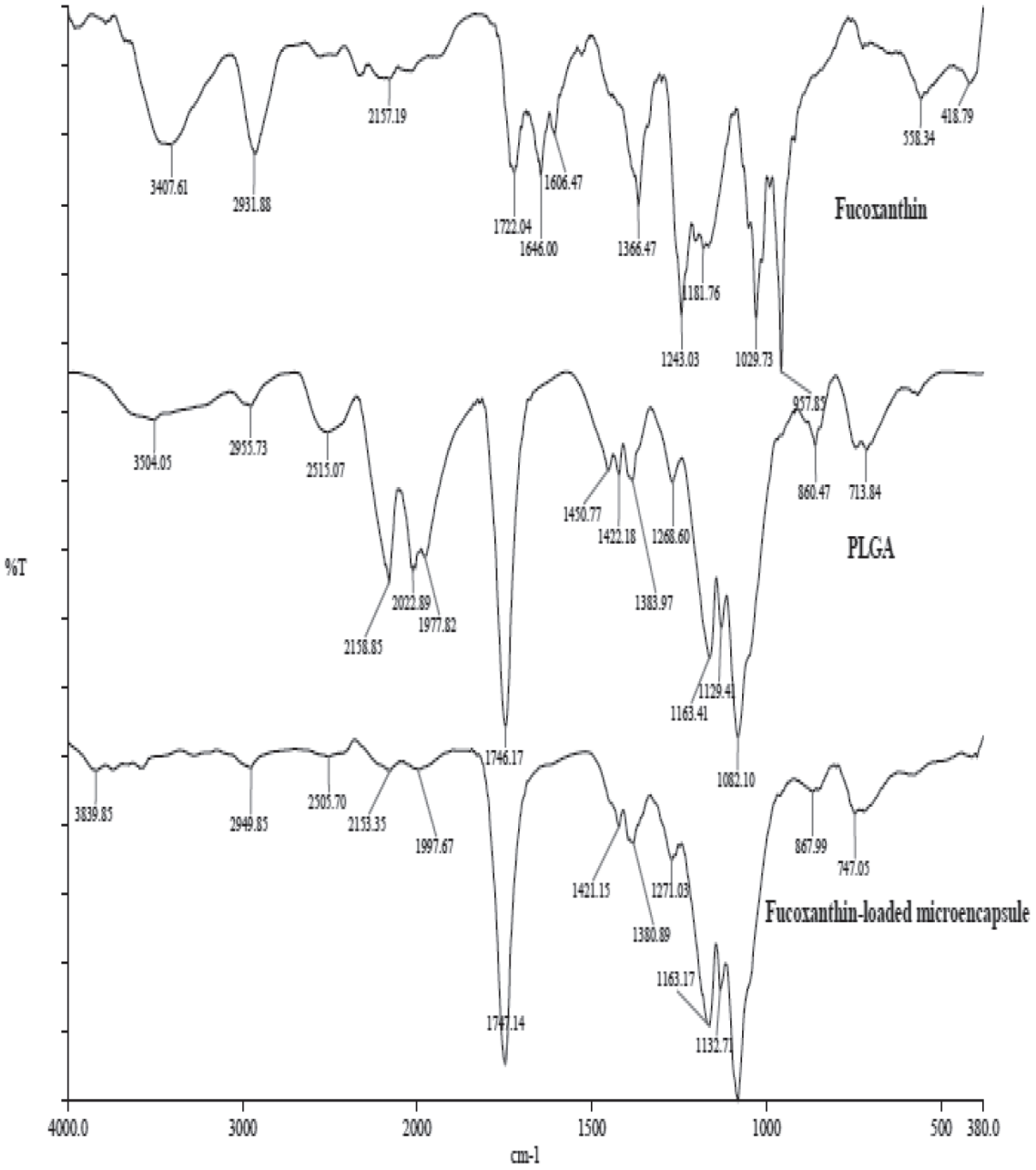

Fig. 13 FTIR spectra of fucoxanthin, PLGA and F-LM. ATR-FTIR spectrometer (Spectrum 100, Perkin Elmer) was used to analysis of functional groups of the materials.

trapped in the PLGA microsphere. Additionally, fucoxanthin (as core) maintained good stability during the preparation process. From the characteristic peaks of XRD pattern, it can be concluded that fucoxanthin was successfully encapsulated by PLGA as expected.

4 Conclusion

Fucoxanthin was microencapsulated in PLGA using a two- step w/o/w double-emulsion solvent extraction method to produce fucoxanthin- loaded microspheres (F-LM). Microencapsulation is a promising approach for drug delivery to protect against rapid degradation and allow controlled release during administration. The results of this study suggest that type of surfactant, homogenization speed, concentration of the PLGA polymer and concentration of the surfactant were significant processing parameters affecting size and surface morphology of the final F-LM. Among the surfactants, PVA showed best results in terms of size and surface morphology, while Span-20 was least desirable. Higher homogenization speeds resulted in

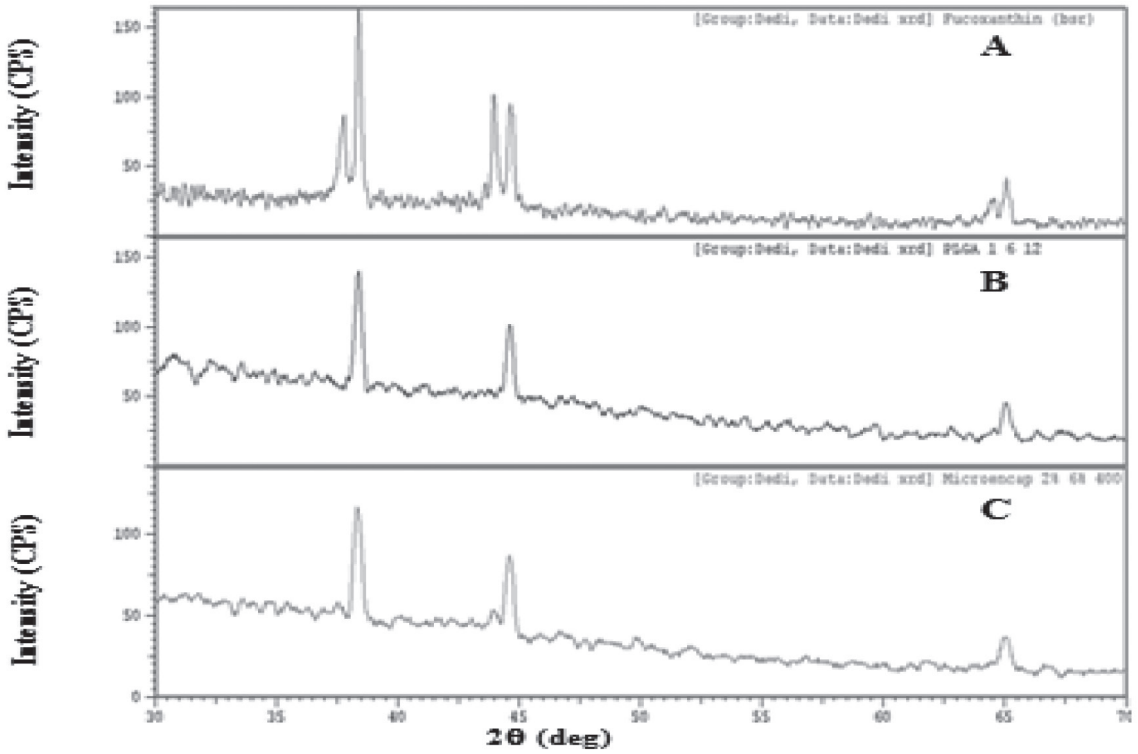

Fig. 14 XRD spectra of fucoxanthin (A), PLGA (B) and F-LM (C). Samples were evaluated using XRD (Shimadzu, XRD-6000 model, Japan) with X-ray tube: Cu (1.54060 A); voltage: 40.0 kV; current: 30.0 mA; step size: 0.0200 deg; and scan range: 30 – 70 deg, 2θ angle.

better F-LM products compared to lower speeds. Concentration effect of PVA and PLGA had opposite effects on the F-LM; higher concentrations of PVA produced smaller particles, while higher concentrations of PLGA produced more desirable microspheres. Characterization of the product before and after microencapsulation can be carried out to evaluate the success of a microencapsulation procedure. Thermal behavior analyses of F-LM carried out using TGA and DSC, as well as functional group analysis using ATR-FTIR and crystallinity analysis using XRD indicated that fucoxanthin was successfully entrapped in the PLGA matrix whilst maintaining its chemical and structural integrity as a core. The results present a two- step w/o/w double-emulsion solvent extraction method for the fabrication of fucoxanthin-loaded microspheres as a drug delivery system for potential applications in pharmacy and biomedicine.

Acknowledgments

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References

1) McNamee, B. F.; O'Riordan, E. D.; O'Sullivan, M. Emulsification and microencapsulation properties of gum Arabic. *J. Agric. Food Chem.* **46**, 4551-4555 (1998).
2) Champagne, C. P.; Fustier, P. Microencapsulation for the improved delivery of bioactive compounds into foods. *Curr. Opinion Biotechnol.* **18**, 184-190 (2007).
3) AppaRao, B.; Shivalingam, M. R.; Reddy, Y. V. K.; Sunitha, N.; Jyothibasu, T.; Shyam, T. Design and evaluation of sustained release microencapsules containing diclofenac sodium. *Int. J. Pharmaceutic. Biomed. Res.* **1**, 90-93 (2010).
4) Wischke, C.; Borchert, H. H. Influence of the primary emulsification procedure on the characteristics of small protein-loaded PLGA microparticles for antigen delivery. *J. Microencapsul.* **23**, 435-448 (2006).
5) Athanasiou, K. A.; Niederauer, G. G.; Agrawal, C. M. Sterilization, toxicity, biocompatibility and clinical applications of polylactic acid/polyglycolic acid copolymers. *Biomaterials* **17**, 93-102 (1996).
6) Varde, N. K.; Pack, D. W. Microspheres for controlled release drug delivery. *Expert Opin. Biol. Theraphy* **4**, 35-51 (2004).
7) Çirpanli, Y.; Robineau, C.; Çapan, Y.; Çaliş, S. Etodolac loaded poly (lactide-co-glycolide) nanoparticles: formulation and in vitro characterization. *Hacettape*

Univer. J. Fac. Pharm. **29**, 105-114 (2009).
8) Emami, J.; Hamishehkar, H.; Najafabadi, A. R.; Gilani, K.; Minaiyan, M.; Mahdavi, H.; Mirzadeh, H.; Fakhari, A.; Nokhodchi, A. Particle size design of PLGA microspheres for potential pulmonary drug delivery using response surface methodology. *J. Microencapsul.* **26**, 1-8 (2009).
9) Makadia, H. K.; Siegel, S. J. Poly Lactic-co-Glycolic Acid (PLGA) as biodegradable controlled drug delivery carrier. *Polymers* **3**, 1377-1397 (2011).
10) Yang, Y. Y.; Chung, T. S; Ng, N. P. Morphology, drug distribution, and *in vitro* release profiles of biodegradable polymeric microspheres containing protein fabricated by double-emulsion solvent extraction/evaporation method. *Biomaterials* **22**, 231-241 (2001).
11) Chaudhari, K. R.; Shah, N.; Patel, H.; Murthy, R. Preparation of porous PLGA microspheres with thermoreversible gel to modulate drug release profile of water-soluble drug: Bleomycin sulphate. *J. Microencapsul.* **27**, 303-313 (2010).
12) Cui, F.; Cun, D.; Tao, A.; Yang, M.; Shi, K.; Zhao, M.; Guan, Y. Preparation and characterization of melittin-loaded poly (DL-lactic acid) or poly (DL-lactic-co-glycolic acid) microspheres made by the double emulsion method. *J. Control. Release* **107**, 310-319 (2005).
13) Jaraswekin, S.; Prakongpan, S.; Bodmeier, R. Effect of poly (lactide-co-glycolide) molecular weight on the release of dexamethasone sodium phosphate from microparticles. *J. Microencapsul.* **24**, 117-128 (2007).
14) Bhatt, Y.; Shah, D. Influence of additives of Fabrication and Release from Protein loaded PLGA microparticles. *J. Chem. Pharmaceutic. Res.* **4**, 1780-1715 (2012).
15) Manca, M. L. Chitosan and PLGA microspheres as drug delivery system against pulmonary micobacteria infections. A PhD Thesis. Dept. of Farmaco Chimico Technologico. University of Cagliari. 134 p. (2009).
16) Mainardes, R. M.; Evangelista, R. C. Praziquantel-loaded PLGA nanoparticles: preparation and characterization. *J. Microencapsul.* **22**, 13-24 (2005).
17) Freitas, S.; Merkle, H. P.; Gander, B. Microencapsulation by solvent extraction/evaporation: Reviewing the state of the art of microsphere preparation process technology. *J. Control. Release* **102**, 313-332 (2005).
18) Bonnet, M.; Canseli, M.; Placin, F.; David-Briand, E.; Anton, M.; Leal-Calderon, F. Influence of ionic complexation on release rate profiles from multiple water-in-oil-in-water (W/O/W) emulsions. *J. Agric. Food Chem.* **58**, 7762-7769 (2010).
19) Mohamed, F.; Walle, C. F. V. D. PLGA microcapsules with novel dimpled surfaces for pulmonary delivery of DNA. *Int. J. Pharm.* **1**, 97-107 (2006).
20) Ismail, A. F.; Abdalmonemdoolaanea; Awang, M.; Mohamed, F. High initial burst release of gentamicin formulated as PLGA microspheres implant for treating orthopaedic infection. *Int. J. Pharm. Sci.* **4**, 658-691 (2012).
21) Alfatama, M.; Ahmad, K.; Mohamed, F. Microencapsulation of *Cassia alata*: Fabrication and characterization. *Int. J. Pharm. Sci.* **4**, 219-224 (2012).
22) Mohamed, H. F. Formulation and evaluation of polyester microspheres by solvent- evaporation method. A PhD Thesis. Strathclyde Institute of Pharmacy and Biomedical Science. University of Strathclyde. 349 p (2008).
23) Karatas, A.; Sonakin, O.; Kilicarslan, M.; Baykara, T. Poly (epsilon-caprolactone) microparticles containing Levobunolol HCl prepared by a multiple emulsion (W/O/W) solvent evaporation technique: Effects of some formulation parameters on microparticle characteristics. *J. Microencapsul.* **26**, 63-74 (2009).
24) Brunner, C. T.; Baran, E. T.; Pinho, E. D.; Reis, R. L.; Neves, N. M. Performance of biodegradable microcapsules of poly (butylenes succinate), poly (butylenes succinate-co-adipate) and poly (butylenes terephthalate-co-adipate) as drug encapsulation systems. *Coll. Surfaces B. Biointerface* **84**, 498-507 (2011).
25) Lekshmi, U. M. D.; Poovi, G.; Reddy, P. N. *In vitro* observation of repaglinide engineered polymeric. *Dig. J. Nanomater. Biostruct.* **7**, 1-18 (2012).
26) Alagusundaram, M.; Madhu, S. C. C.; Umashankari, K.; Attuluri, V. B.; Lavanya, C.; Ramkanth. S. Microspheres as a novel drug delivery system – a review. *Int. J. Chem. Tech. Res.* **1**, 526-534 (2009).
27) Zainudin, B. M. Therapeutic aerosol: principles and practices. *Med. J. Malaysia* **48**, 259-268 (1993).
28) Meenach, S. A.; Kim, Y. J.; Kauffman, K. J.; Kanthamneni, N.; Bachelder, E. M.; Ainslie, K. M. Synthesis, optimization, and characterization of camptothecin-loaded acetalated dextran porous microparticles for pulmonary delivery. *Mol. Pharm.* **9**, 290-298 (2012).
29) Mu, L.; Feng, S. S. Vitamin E TPGS used as emulsifier in the solvent evaporation/extraction technique for fabrication of polymeric nanospheres for controlled release of paclitaxel (Taxol®). *J. Control. Release* **80**, 129-144 (2002).
30) Hong, J.; Hong, C. K.; Shim, S. E. Synthesis of polystyrene microspheres by dispersion polymerization using poly (vinyl alcohol) as a steric stabilizer in aqueous alcohol media. *Coll. Surfaces A: Physicochem. Eng. Aspects.* **302**, 225-233 (2007).
31) Keegan, M. E. Biodegradable Microspheres with Enhanced Capacity for Surface Ligand Conjugation. A PhD thesis. Faculty of the Graduate School of Cornell University. 118 p. (2004).
32) Zhu, K. J.; Jiang, H. L.; Du, X. Y.; Wang, J.; Xu, W. X.; Liu, S. F. Preparation and characterization of hCG-loaded polylactide or poly (lactide-co-glycolide) micro-

spheres using a modified water-in-oil-in-water (w/o/w) emulsion solvent evaporation technique. *J. Microencapsul.* **18**, 247-260 (2001).
33) Fu, J.; Fiegel, J.; Krauland, E.; Hanes, J. New polymeric carriers for controlled drug delivery following inhalation or injection. *Biomaterials* **23**, 4425-4433 (2002).
34) Kushwaha, P.; Fareed, S.; Nanda, S.; Mishra, A. Design & fabrication of tramadol HCl loaded multiparticulate colon targeted drug delivery system. *J. Chem. Pharm. Res.* **3**, 584-595 (2011).
35) Hong, Y.; Gao, C.; Shi, Y.; Shen, J. Preparation of porous polylactide microspheres by emulsion-solvent evaporation based on solution induced phase separation. *Polym. Adv. Technol.* **16**, 622-627 (2005).
36) Ehtezazi, T.; Washington, C.; Melia, C. D. Determination of the internal morphology of poly (D,L-lactide) microspheres using stereological methods. *J. Contr. Rel.* **32**, 231-241 (1999).
37) Mehrotra, A.; Pandit, J. K. Critical Process Parameters Evaluation of Modified Nanoprecipitation Method on Lomustine Nanoparticles and Cytostatic Activity Study on L132 Human Cancer Cell Line. *J. Nanomed. Nanotechnol.* **3**, 1-8 (2012).
38) Pradeesh, T. S.; Sunny, M. C.; Varma, H. K.; Ramesh, P. Preparation of microstructured hydroxyapatite microspheres using oil in water emulsions. *Bull. Mater. Sci.* **28**, 383-390 (2005).
39) Ribeiro-Costa, R. M.; Alvest, A. J.; Santos, N. P.; Nascimentos, S. C.; Gonçalves, E. C. P.; Silva, N. H.; Honda, N. K.; Santos-Magalhaes. *In vitro* and *in vivo* properties of usnic acid encapsulated into PLGA-microspheres. *J. Microencapsul.* **21**, 371-384 (2004).
40) Obeidat, W. M. Recent patents review in microencapsulation of pharmaceuticals using the emulsion solvent removal methods. *Recent Patents Drug Deliv. Formul.* **3**, 178-192 (2009).
41) Milanovic, J.; Manojlovic, V.; Levic, S.; Rajic, N.; Nedovic, V.; Bugarski, B. Microencapsulation of flavors in Carnauba wax. *Sensrs.* **10**, 901-912 (2010).
42) Schnitzler, E.; Lençone, K.; Kobelinik, M. Characterization of Pharmaceutical by thermal analysis. *Exact Soil Sci. Agrarian S Eng. S.* **1**, 91-100 (2002).
43) Prasanth, V. V.; Moy, A. C.; Mathew, S. T.; Rinku, M. R. Microspheres-An Overview. *Int. J. Res. Pharmacetic. Biomed. Sci.* **2**, 332-338 (2011).
44) Guirguis, O. W.; Moselhey, M. T. H. Thermal and structural studies of poly (vinyl alcohol) and hydroxypropyl cellulose blends. *Nat. Sci.* **4**, 57-67 (2012).
45) Dubernet, C. Thermoanalysis of microspheres. *Thermochim. Acta.* **248**, 259-269 (1995).
46) Blasi, P.; D'Souza, S. S.; Selmin, F.; DeLuca, P. P. Plasticizing effect of water on poly (lactide-co-glycolide). *J. Control. Release* **108**, 1-9 (2005).
47) Bouissou, C.; Walle, C. F. V. D. Poly (lacticco-glycolic acid) Microspheres. In Uchegbu, I. F.; Schatzlein, A. G. (Eds.), Polymers in Drug Delivery (1st ed.) New York: CRC Press. pp. 81-94 (2006).
48) Wang, Y. pH-Sensitive and targeted PLGA-based drug delivery to colorectal cancer. A PhD thesis. Deakin Univ. 192 p. (2012).
49) Passerini, N.; Craig, D. Q. M. An investigation into the effects of residual water on the glass transition temperature of polylactide microspheres using modulated temperature DSC. *J. Control. Release* **73**, 111-115 (2001).
50) Li, S.; Vert, M. Biodegradable polymers: Polyesters. In: The Encyclopedia of controlled drug delivery. Mathiowitz, E. (Ed.). John Wiley & Sons, New York. pp. 71-93 (1999).
51) Gaisford, S.; Asma, B.; Buanz, M. Pharmaceutical physical form characterization with fast (>200℃ min-1) DSC heating rates. *J. Therm. Anal. Calorim.* **106**, 221-226 (2011).
52) Yockman, J. W.; Maheshwari, A.; Han, S.-O.; Kim, S. W. Tumor regression by repeated intratumoral delivery of water soluble lipopolymers/p2CMVmIL-12 complexes. *J. Control. Res.* **87**, 177-186 (2003).
53) Sahil, K.; Akanksha, M.; Premjeet, S.; Bilandi, A.; Kapoor, B. Microsphere: a review. *Int. J. Res. Pharm. Chem.* **1**, 1184-1198 (2011).
54) Surini, S.; Anggraini, V.; Anwar, E. Study of Mucoadhesive Microspheres Based on Pregelatinsed Cassava Starch Succinate as a New Carrier for Drug Delivery. *J. Med. Sci.* **9**, 249-256 (2009).
55) Yamaguchi, Y.; Takenaga, M.; Kitagawa, A.; Ogawa, Y.; Mizushima, Y.; Igarashi, R. Insulin-loaded biodegradable PLGA microcapsules: Initial burst release controlled by hydrophilic additive. *J. Contr. Release* **81**, 235-249 (2002).